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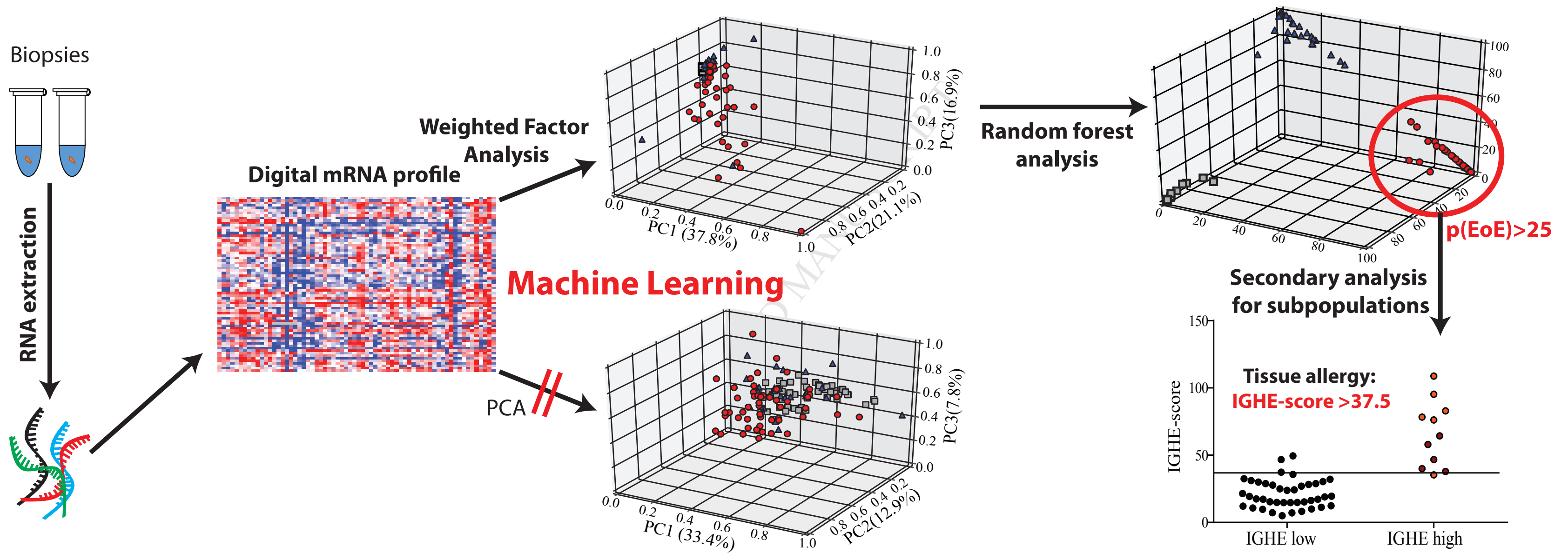
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**An algorithm for the classification of mRNA patterns in eosinophilic esophagitis:
integration of machine learning**

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Abstract (250)

Background: Diagnostic evaluation of eosinophilic esophagitis (EoE) remains difficult, particularly the assessment of the patient's allergic status.

Objective: Establish an automated medical algorithm to assist in the evaluation of EoE.

Methods: Machine learning techniques were used to establish a diagnostic probability score for EoE (pEoE) based on esophageal mRNA transcript patterns from biopsies of patients with EoE, gastroesophageal reflux disease, and controls. Dimensionality reduction in the training set established weighted factors, which were confirmed by immunohistochemistry. Following weighted factor analysis, p(EoE) was determined by Random Forest classification. Accuracy was tested in an external test set and predictive power was assessed with equivocal patients. Esophageal IgE production was quantified with epsilon germ line (IGHE) transcripts and correlated with serum IgE and the Th2-type mRNA profile to establish an IGHE-score for tissue allergy.

Results: In the primary analysis, a three-class statistical model generated a p(EoE) score based on common characteristics of the inflammatory EoE profile. A p(EoE) ≥ 25 successfully identified EoE with high accuracy (sensitivity 94.4%, specificity 92.9%, AUC 0.985) and improved diagnosis of equivocal cases by 84.6%. The p(EoE) changed in response to therapy. A secondary analysis loop in EoE patients defined an IGHE-score ≥ 37.5 for a patient subpopulation with increased esophageal allergic inflammation.

Conclusion: The development of intelligent data analysis from a machine learning perspective provides exciting opportunities to improve diagnostic precision and improve patient care in EoE. The p(EoE) and the IGHE-score are steps towards the development of decision trees to define EoE subpopulations and, consequently, will facilitate individualized therapy.

Key Messages

A diagnostic algorithm based on esophageal mRNA transcript patterns was developed utilizing machine learning techniques to diagnose EoE with high precision. This strategy was also used to identify a subgroup of EoE patients characterized by IgE-mediated tissue allergy, which may allow for a personalized treatment approach.

Capsule summary (35 words)

A medical algorithm uses esophageal mRNA patterns to diagnose EoE with a high level of competence. Esophageal IgE production was used to assess the allergic status providing a step towards individualized medicine and patient-oriented outcome.

Key words (up to 10)

- Allergy diagnosis
- Eosinophils
- Eosinophilic esophagitis
- Chronic allergic inflammation
- IgE
- Machine learning
- Medical algorithm

Abbreviations

AUC, area under the curve

CPA3, carboxipeptidase A3

EGID, eosinophilic gastrointestinal disorder

- 89 EoE, eosinophilic esophagitis
- 90 GERD, gastroesophageal reflux disease
- 91 IGHE, epsilon germline transcripts
- 92 MC, mast cells
- 93 p(EoE), diagnostic probability score for EoE

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Authors' contributions

BFS, LE, SMR, UA, WAD, LAS, and EF designed experiments, performed research, and analyzed data. UA, RW, WSL, ZS, JT, JDG, SBS, JAV, and SN were involved in patient recruitment, evaluation of clinical patient status, evaluation of biopsies, and analysis of human serum data. BFS, SN, and EF wrote the first draft of manuscript. All co-authors contributed to writing the final manuscript and approved its last version.

Introduction

Eosinophilic esophagitis (EoE) is classified as a primary eosinophilic gastrointestinal disorder (EGID) ¹⁻⁶. The unifying hallmark and diagnostic marker of all EGIDs is an eosinophil-rich inflammatory infiltrate of the affected mucosa as determined by histology ^{4, 7-10}. The etiologies of EGIDs are generally not well understood ¹¹. Tissue eosinophilia is typically considered of unknown origin and disease pathogenesis are believed to involve a complex interplay of genetic predisposition, exposure to food and/or environmental allergens, and involves IgE-mediated activation of the immune system ^{6, 9, 12-14}.

Currently, quantification of tissue eosinophils (>15 eosinophils per high power field) in combination with assessment of clinical symptomatology is the gold standard for identifying patients with EoE ^{12, 14-18}. The histologic diagnostic strategies have been consistently improved ¹⁹ but do not account for subtypes of disease ^{20 21}. The wide variation of therapy responses and the lack of reliable predictors of therapy outcome ²² further stress the need to improve current diagnostic strategies. In conclusion, clinical assessment of EoE remains difficult and efforts to establish adjunct strategies to improve diagnosis, to facilitate monitoring of disease progression, and to predict therapy outcome are warranted ^{12 18}.

EoE has been defined as an allergic immune disorder characterized by eosinophil-rich, chronic Th2-type inflammation of the esophagus with fibrosis resulting in esophageal strictures and dysphagia as long-term complications ^{7, 9, 13, 14, 23}. The predominant tissue-specific esophageal pathology of EoE is, at least in part, explained by the observation that tissue differentiation of the esophagus is severely impaired in EoE patients ^{6, 24}. Although EoE is frequently associated with other IgE-mediated conditions that are not restricted to the esophagus such as allergic asthma, atopic dermatitis and/or food allergy, assessment of the IgE-mediated allergic status in relation to EoE patient proves challenging ^{12, 25}. This is because total serum IgE levels and IgE-specific allergen titers correlate poorly with EoE ^{25, 26}. An effect of allergic comorbidities on EoE has been demonstrated for patients with coexisting food allergy ²⁷, but not necessarily with classical IgE-mediated forms of food allergy ²⁸. At this point, the interplay between different types of allergies and how it affects EoE requires more attention ^{14, 26, 29}. Diagnostic parameters to unequivocally define EoE patients that suffer from a classical IgE-mediated allergy or IgE-independent types of EoE are currently not available.

152 An EoE-specific esophageal transcriptome has been defined more than a decade
153 ago³⁰ and, since then, mRNA-based strategies have been analyzed for their potential to
154 improve EoE diagnosis and patient care. Establishing a PCR-based EoE scoring system
155³¹ and defining diagnostic digital mRNA pattern stamps^{32, 33} were important first steps in
156 this research area. The remaining challenges are now to translate published
157 observations to platforms that are easily accessible to the broad public and their health
158 care providers. In this regard, a medical algorithm as a broadly accessible clinical
159 decision support system could provide an important step towards improving
160 personalized care for EoE patients^{22, 34}.

161 We have established a predictive EoE probability score ($p(\text{EoE})$) based on a
162 medical algorithm that uses targeted esophageal mRNA transcript analysis. The
163 establishment of an mRNA pattern-based diagnostic approach in combination with
164 machine learning techniques for EoE is a strategy in line with the NIH Roadmap initiative
165 goal of the application of innovations in bioinformatics to bedside clinical practice^{35 36}.
166 The $p(\text{EoE})$ provides a promising tool to facilitate primary diagnosis of EoE and an
167 important step towards establishing strategies for personalized therapy based on
168 individual inflammatory characteristics of subtypes of disease.

169

METHODS

Study population

Over the last 9 years, a patient cohort for studying the pathology of EoE and other esophageal disorders has been established at the EGID Center of BCH (<http://www.childrenshospital.org/centers-and-services/eosinophilic-gastrointestinal-disease-program>). The recruitment details for the patient population have been published^{32, 37, 38}. Study approval was obtained from the Institutional Review Board of Boston Children's Hospital (Boston, MA, approval #07-11-0460). All patients or their legal guardians provided written consent prior to enrollment.

The registry contains individuals suffering from EoE, gastroesophageal reflux disease (GERD), and controls as defined by the absence of esophageal inflammation. Study biopsies were obtained from the proximal and the distal esophagus defined as lying either ≥ 10 cm or 1-2 cm from the gastroesophageal junction respectively. Biopsies were immediately stored in RNeasy Lysis Buffer (RLTB, Qiagen) and frozen at -80°C for a minimum of 24h. Digital mRNA pattern stamps were generated from both biopsies using the nCounter[®] system (Nanostring technology (nCounter[®] system, www.nanostring.com,³⁹) as published by the lab previously³². In brief, specimens were homogenized in RLTplus buffer (Qiagen) and processed with the nCounter[®] Prep Station and Digital Analyzer following the manufacturer's instructions. Samples were analyzed using a panel of 79 target probes consisting of five internal positive controls, five housekeeping genes, and 69 probes customized for analyzing expression levels of mRNA transcripts based on the published EoE transcriptome (^{30, 32}, list of targets in Table E1 in online repository).

Clinicopathological diagnosis by reference standards

Board-certified pediatric gastroenterologists who were blinded to the results of mRNA pattern profiling reviewed the clinicopathological diagnosis of each patient. Following consensus guidelines, patients were diagnosed with EoE when they met the following criteria: 1) treatment with PPI for ≥ 4 weeks prior to diagnostic endoscopy, 2) tissue eosinophil count $>15/\text{hpf}$ in at least one biopsy, and 3) exclusion of other origins of esophageal eosinophilia. Use of corticosteroids was considered as exclusion criteria. Patients were classified as GERD when they showed: 1) histological evidence of esophageal tissue inflammation such as basal zone hyperplasia and an inflammatory cell infiltrate, 2) eosinophil count 1-15/hpf, 3) a clinical history suggestive of reflux-

associated symptoms, 4) evidence of GERD either by abnormal pH/impedance studies, or by erosive esophagitis that healed after antacid therapy, and 5) no evidence of development of EoE after follow-up. Control patients were defined as having 1) normal tissue histology in all biopsies and 2) no evidence of underlying esophageal disease for at least three months after endoscopy in the absence of antacid therapy. Patients that did not meet the three diagnostic categories were excluded from the training and test set and used for the equivocal test set. Note that the operator was blinded to the patient diagnosis of the test set and the equivocal patient set for all computer-based analysis.

Assessment of clinical allergy and allergic status of the patients has been performed according to clinical standards as published by the research group^{38, 40}.

Medical algorithm for establishing a predictive EoE score based on mRNA transcript patterns

The application used for the automatization of the data analysis was written in python 3.5.2 and executed using the Anaconda 4.3.11 Python distribution (<https://anaconda.org/>) and Jupyter Notebook 4.1.1. The modules used were scipy 0.18.1, numpy 1.11.1, pandas 0.18.1, scikit-learn 0.18, and matplotlib 1.5.3.

nCounter®-derived mRNA patterns of individual patients were normalized to the sum of the geometric mean of the internal positive controls and the housekeeping genes as recommended by the manufacturer. Patients whose positive control normalization factor was outside the range of 0.33-3 or whose housekeeping gene normalization factor was outside the range of 0.1-10 were excluded. Next, the dimensionality of the normalized transcripts was reduced by weighted component analysis to generate six factors representing the difference between two of the possible diagnostic outcomes. These factors are referred to as $EoE/Control^{dis}$, $EoE/GERD^{dis}$, $GERD/Control^{dis}$, $EoE/Control^{prox}$, $EoE/GERD^{prox}$, and $GERD/Control^{prox}$. The significance (p-value) between the mRNA transcripts of two conditions (e.g. EoE and controls) was calculated by Mann-Whitney U tests. Then, the weight of individual mRNA transcripts was calculated as the product of the $-\log_{10}(p\text{-value})$ between the two diagnoses and the $\log_2(\text{fold difference})$ between them.

The dimensionality-reduced data set was then applied to build a statistical model of diagnosis using Random Forest classification to determine probability scores for belonging to one of the diagnostic groups ($p(EoE)$, $p(GERD)$, $p(Control)$). Random decision trees were trained on the gene expression data of the training set and bagged

to reduce the chance of over fitting using the sklearn.ensemble plugin for python. The cut off for EoE diagnosis was determined by ROC analysis as $p(\text{EoE}) \geq 25$. The accuracy was evaluated with the external test set and with a second test set of the equivocal patients. A secondary analysis loop using weighted factor analysis in EoE patients was used to define the IGHE score.

Immunohistochemistry and quantification

Paraffin-embedded distal esophageal tissue sections from EoE, controls, and GERD patients were deparaffinized and rehydrated. Heat antigen retrieval treatment was performed at 100°C for 30 minutes. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide/PBS for 5 minutes. Sections were blocked with PBS with 10% goat serum for 30 minutes and incubated with either anti-HIF1A (LSBio, 74257) (1:50) overnight at 4°C. Goat anti-rabbit HRP (1:100) was applied and incubated for 30 minutes followed by detection with Vectastain® ABC peroxidase system (Vector-labs, Peterborough, UK) according to the manufacturer's protocol and visualized with DAB reagent (Vector-labs, Peterborough, UK). Sections were counterstained with hematoxylin (Vector-labs, Peterborough, UK). Pictures were taken at 200x or 400x magnifications with Olympus DP70 microscope. Area quantification was performed with ImageJ by separating the color channels using the color deconvolution plugin. Color thresholds were set using a negative control staining. The defined area of the stained tissue was measured.

Statistical analysis

Comparison of continuous variables between diagnostic groups was performed by Kruskal-Wallis test, using Dunn's multiple comparison post-hoc tests, when appropriate. Fisher's exact test was used for dichotomous predictors. Correlation analysis was performed using Spearman rank correlation coefficients. Values are expressed as mean $\pm$ SEM. Analyses were performed using IBM SPSS Statistics 23 (IBM Corp. 7, Armonk, NY), or GraphPad Prism 7 (GraphPad, San Diego, California).

RESULTS

Application of machine learning strategies for identifying EoE patients

We selected mRNA expression patterns of 226 patients from a cohort study on esophageal inflammation recruited between July 2008 and November 2015 (Fig 1, A). 33 patients were excluded because they failed normalization criteria or only one biopsy was available. Of the remaining 193, 79 patients (40.9%) had normal histology and were classified as controls. 114 patients (59.1%) presented with histological evidence of esophageal inflammation. Using the gold standard of diagnosis, 68/114 patients (59.65%) were diagnosed with EoE and 46/114 patients (40.3%) were diagnosed with GERD. 13/114 patients (11.4%) could not be diagnosed based on the histological evaluation of the biopsies obtained at their first visit and were placed in the equivocal test set. Detailed patient characteristics are summarized in Table I.

An attempt to predict EoE with principal component analysis as an unsupervised machine learning approach did not yield satisfying separation of patient groups (Fig E1 in the online repository). To improve the diagnostic power, we developed a three-class statistical model based on supervised machine learning techniques. The dimension of the normalized mRNA transcript data sets was reduced to six factors each representing the difference between two of the three possible conditions in the proximal or the distal biopsies (Fig 1, B for EoE/GERD^{prox}, EoE/GERD^{dis}; and Fig E2 in the online repository). During this process, individual transcripts were automatically assigned weights that affected the calculation of the factors greatly if weighted high or proportionally less if weighted low (Fig 1, B-C). In line with the literature, the program weighted transcripts highly that had been published as EoE markers (e.g.: eotaxin, periostin, and Carboxipeptidase A3 (CPA3)^{15, 30}) for factors that distinguish EoE from GERD or Controls (Fig 1, B). Accordingly, transcripts that are expressed independently of EoE were assigned low weights (e.g.: Galectin-3^{32, 38}, Fig 1, C).

Immunohistochemistry confirms weighing strategy on the protein level

To independently confirm the results of the computer program and to test if absence of Hypoxia-inducible factor 1-alpha (HIF1A) could be used as a single protein marker to facilitate identification of EoE patients, immunohistochemistry of esophageal biopsies was performed (Fig 2, A and B). In correlation to results of the dimensionality reduction, HIF1A protein levels were lower in EoE than in control or GERD biopsies.

Quantification of the slides with ImageJ confirmed comparatively low expression levels of HIF1A in EoE (Fig 2, C). This set of data provides an independent confirmation for the computer-based weighting strategy on the protein level.

Establishing probability scores

A principal component analysis of the weighted factors improved the clustering of the diagnostic groups but still did not provide satisfying separation (Fig E3 in online repository). Random Forest classification as an alternative supervised approach was used to establish disease-specific probability scores that discriminated between the three patient categories (Fig 3, A and B) with a mean predictive score of $p(\text{EoE})$ in EoE patients (91 ± 1 , range 68-100), $p(\text{GERD})$ in GERD patients (86 ± 2 , range 67-98), and $p(\text{Control})$ in control patients (96 ± 1 , range 79-100). Cluster analysis confirmed that this diagnostic model separated the population in the training set based on the underlying clinical diagnosis (Fig 3, C).

Testing diagnostic accuracy and predictive power of $p(\text{EoE})$

To determine a cutoff for EoE diagnosis and the sensitivity and specificity of $p(\text{EoE})$, the algorithm was applied to the external patient test set in a blinded fashion (Fig 4, A). Using ROC analysis the optimal cutoff for EoE diagnosis was determined as $p(\text{EoE}) \geq 25$ (Fig 4, B and C). This cutoff distinguished EoE patients with a sensitivity of 0.91 and a specificity of 0.93 with an AUC of 0.985. Analysis of individual transcripts demonstrated that expression of EoE signature genes, such as CCL26 and periostin, correlates strongly with EoE. ROC analysis, however, showed that single gene analysis cannot separate patient groups with the accuracy of the $p(\text{EoE})$ (Fig E4 in the online repository).

To determine the predictive power of the approach, we next tested $p(\text{EoE})$ in the equivocal patient set ($n=13$, Fig 1, A and Table I). Using the first equivocal biopsies, the algorithm predicted EoE or GERD with an accuracy of 84.6% as later established during a minimum of two follow-up clinic visits (Fig 4, D). Thus, this medical algorithm allows for the accurate diagnosis of esophageal inflammatory disorders in a substantial number of equivocal patients that could not be classified by current diagnostic standard at the time of their first biopsy.

Therapy response is reflected in alterations of the p(EoE)

Currently, eosinophil counts are used to measure of disease severity/activity in EoE and therapy response is monitored by the reduction of tissue eosinophilia. We first demonstrated that the p(EoE) correlates with tissue eosinophil counts and eotaxin transcripts (Fig E5 in online repository) and next analyzed how p(EoE) is altered in response to therapy. Esophageal mRNA pattern stamps from therapy responsive EoE patients were collected before and after steroid treatment to calculate the p(EoE). In 4/5 patients eosinophilia resolved completely (Eos/HPF<5) and, accordingly, the p(EoE) dropped below the cut off for EoE diagnosis (<25). In one patient, eosinophil counts dropped (5<Eos/HPF<15) but eosinophilia did not resolve completely. Accordingly, the p(EoE) dropped substantially but remained >25 (Fig 5, A). Single transcript analysis further showed that therapy response resulted in downregulation of heavily weighted upregulated genes (e.g. CCL26 in Fig 5, B and C) while transcript numbers of genes that were negatively regulated in EoE were induced (e.g. HIF1A in Fig 5, B and C). This set of data demonstrates that the p(EoE) can be used as a diagnostic tool to monitor disease severity and therapy response.

Secondary analysis for quantification of local IgE production and tissue allergy

EoE is considered a chronic allergic disease of the esophagus but levels of total IgE, the classical marker of allergy, are commonly not found elevated in serum independently of atopic comorbidities^{26,41}. When stratifying our cohort into groups of patients with and without clinically diagnosed allergies, we observed no significant difference in peak eosinophil count or p(EoE) (Fig E6 in the online repository). In our cohort, only 45.5% of EoE patients present with elevated IgE (Table I). Dimensionality reduction depicted esophageal 3' IGHE germline transcripts, a surrogate for local IgE production by tissue resident B cells⁴², as one of the highest weighted factors in differentiating EoE from GERD and controls. We thus asked whether local IgE production could be used to establish a score for the severity of IgE-mediated esophageal Th2-type allergic inflammation. Using mean IGHE+2 SEM in controls and GERD as cutoff (horizontal line in Fig 6, A), EoE patients were divided into an IGHE-high and an IGHE-low patient group. The occurrence of food allergies was significantly higher in IGHE-high EoE patients when compared to IGHE-low with non-elevated serum IgE but the occurrence of food allergy was comparable between IGHE-high EoE and IGHE-low patients with elevated serum IgE (for additional clinical characteristics and incidence

of allergic comorbidities see Table E2). IGHE expression and serum IgE levels did not correlate (Spearman rank $r = 0.14$, $p=0.30$; Fig 6, A). Esophageal eosinophil counts and p(EoE) did not differentiate patients when grouped based on serum IgE titers or IGHE expression (white circle for IGHE-low/serum-IgE-normal, black circles for IGHE-low/serum-IgE-elevated patients; orange IGHE-high/serum-IgE-normal; red IGHE-high/serum-IgE-elevated; Fig 6, B and Fig E7, B in the online repository). Indicative for increased allergic tissue inflammation, the IGHE-high population showed a more pronounced Th2-type inflammatory mRNA profile with significantly higher levels of mast cell marker transcripts (CPA3 and Fc ϵ RI β , mast cell-tropic chemokines (CCL2 and CCL5), and Th2-type cytokines (IL13 and IL5) (Fig 6, C and D, and Fig E7 in the online repository).

We reasoned that the application of a secondary level of analysis to EoE patients using another round of supervised machine learning techniques could extend the utilization of the medical algorithm identifying a subpopulation of EoE patients with strong esophageal IgE-mediated allergic inflammation (Fig 7, A). For this purpose, we established an IGHE score by weighted component analysis of transcript patterns of IGHE-high and IGHE-low EoE patients. Using ROC analysis the optimal cutoff was determined (IGHE-score ≥ 37.5) which resulted in a sensitivity of 0.90 and a specificity of 0.95 for differentiating IGHE-high and IGHE low patients (Fig 7, B and C). As expected for a readout of tissue allergy, the IGHE score correlated highly with typical markers of mast cell mediated inflammation, such as Fc ϵ RI β and CPA3 (Fig 7, D and data not shown). Using the identification of EoE patients with pronounced local esophageal allergic inflammation as an example, this set of data shows that machine learning based diagnostic approaches enable the identification of patient subgroups.

DISCUSSION

For the initial medical management of EoE, providers do not have all of the diagnostic tools to guide directed therapies for several reasons. First and foremost, the diagnostic evaluation of EoE currently strictly relies on eosinophil counts in esophageal biopsies. The gold standard diagnosis of >15 eosinophils per high power field leaves a considerable number of patients as equivocal at time of their first assessment¹⁶. Second, assessing the allergic sensitization status of EoE patients proves difficult because patients commonly present with low serum IgE despite signs of clinical allergies^{25, 26}. Furthermore, current diagnostic strategies do not allow for integration of rapidly emerging concepts, such as the idea of distinct EoE phenotypes, into the process that guides the choice of therapeutic strategies^{20 21}. To address these issues, the current study introduces an automated medical algorithm that has been designed to facilitate the assessment of the EoE pathology using transcriptional inflammatory mRNA profiles of the esophageal biopsy tissue with machine learning strategies.

We took advantage of the established EoE-specific esophageal transcriptome^{30, 31, 43} to develop a method for digitally generating a transcriptional mRNA profile from esophageal tissue biopsies³² that can be applied to supervised machine learning. A computer model was established to generate a diagnostic probability score for EoE ($p(\text{EoE})$) as the outcome parameter for primary diagnosis in first visit biopsies. In histologically unequivocal patients, the $p(\text{EoE})$ defines EoE with high sensitivity and specificity (0.91 and 0.93, respectively) and correlated with eosinophil counts and tissue mRNA levels of CCL26 implying that, comparable to the eosinophil count, the $p(\text{EoE})$ can be used as a surrogate marker for disease severity. Comparative analysis of the $p(\text{EoE})$ pre- and post-treatment further demonstrated that this diagnostic tool can be used to monitor therapy response. Furthermore, the observation that the $p(\text{EoE})$ accurately diagnosed EoE in 84.6% of the equivocal patients in their first biopsies is encouraging because this patient population presents a challenge in clinical practice. In such EoE patients, the $p(\text{EoE})$ could prevent the extensive follow-up visits necessary for primary diagnosis, which, in turn, can reduce the time delay between first visit and start of effective therapy. In conclusion, the $p(\text{EoE})$ provides a promising tool for health care providers and will help to reduce patient morbidity and health care costs with anticipated fewer tests and procedures. Additionally, $p(\text{EoE})$ -based diagnosis could provide an advantage over the currently applied golden diagnostic standard by significantly reducing the numbers of equivocal patients.

Another benefit of using this machine learning approach for generating the p(EoE) is the self-improving potential of the algorithm, which results from the fact that the transcript weights as well as the Random Forest classification are dynamically recalculated each time the algorithm is initiated. Thus, increasing patient numbers will improve diagnostic precision. Furthermore, the algorithm was programmed in modules to assure flexibility and allow for adaptation to findings from discovery oriented research and/or changes in clinical practice. Importantly, the incorporation of novel data is not limited to mRNA transcripts. The diagnostic algorithm can be modified to integrate diverse forms of information such as serum markers or questionnaires as long as they can be expressed numerically. While the addition of more data is unlikely to contribute greatly to the accuracy of the p(EoE), this strategy may be vital to establish additional scores in secondary rounds of analysis to establish a medical decision tree for individualized and outcome-oriented EoE patient care.

Current clinical diagnostic strategies do not account for emerging distinct patient subpopulations of EoE^{20, 21}. With a secondary analysis loop that established an IGHE score as a measure of IgE-mediated allergic local tissue inflammation, we demonstrated that the medical algorithm might be able to address this diagnostic gap. In the absence of antigen-specific IgE titers, the allergic status of individual patients is hard to assess and patients that suffer from IgE-mediated EoE are hard to depict. We show that epsilon germ line transcripts and the correlating Th2-type mRNA markers, which form the basis for the IGHE-score, can be used as a measurable parameter for the esophageal allergic tissue profile when defining the allergic status of a patient. The finding that both mast cell tropic chemokines, CCL2 and CCL5, correlate strongly with local IgE production further imply that IGHE score is an indicator for the extent to which IgE fuels local allergic tissue inflammation. Thus, the IGHE-score might be a valuable strategy to identify EoE patients, which are most suitable for IgE-blocking therapy.

The p(EoE) has exciting potential for defining subpopulations of EoE patients. Most recently, the emerging EoE group with 'proton pump inhibitor responsive' esophageal eosinophilia (PPI-REE) poses a new challenge in clinical practice⁴⁷. The transcriptome of this EoE population has been published⁴³, but the design of the diagnostic mRNA pattern stamp for the p(EoE) precedes this development in the field. The self-learning features of artificial intelligence systems allow for rapid adaptation of algorithms, which will facilitate modifications the established program for defining the p(EoE) to continuously integrate the most recent basic research observations. For

example, the integration of genes with relevance to PPI-REE patients, such as KCNJ2, will likely allow for establishing another loop for the identification of this EoE patient subpopulation.

In summary, the use of a machine learning based approach and medical algorithms in clinical practice offer a number of long term treatment benefits for EoE patients. Going forward, application of the p(EoE) will not only be valuable as a primary diagnostic tool but may also provide a strategy to apply machine learning algorithms to facilitate the direct translation of the expanding knowledge of EoE pathology from bench to bedside. Furthermore, the use of computer-based automated strategies in EoE diagnosis might also help to identify emerging EoE subtypes in order to provide personalized treatment options.

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Figure legends:

Fig. 1: Recruitment and dimensionality reduction of normalized mRNA transcripts.

A, Patient selection. **B**, Determination of gene weights. Volcano plots of normalized mRNA transcripts displayed as fold difference (x-axis) and significance (y-axis) were used for the calculation of the factors differentiating EoE and GERD in the proximal and distal esophagus of the training set n=113. **C**, Transcript weights of the factors differentiating EoE and GERD in the distal biopsy (EoE/GERD^{dis}). Red indicates weight >10.

Fig. 2: HIF1A protein expression is patient biopsies. **A**, Immunohistochemistry of esophageal tissue sections from EoE, Control, and GERD patients stained for HIF1A (n=5 per group; representative distal biopsy). Tissue sections are shown at 200x and 400x. **B**, Isotype control (400x). **C**, ImageJ quantification.; p-values as calculated by Dunn's multiple comparison test after Kruskal-Wallis test

Fig. 3: Establishing diagnostic probability scores. **A**, Training set analysis strategy. **B**, Probability scores displayed as p(EoE), p(GERD), and p(Control). **C**, Cluster analysis based on probability scores.

Fig. 4: Testing diagnostic and predictive accuracy. **A**, External test set and equivocal test set analysis strategy. **B**, ROC analysis of p(EoE) as a diagnostic parameter for EoE, p(Control) as a diagnostic marker for controls, and p(GERD) as a diagnostic marker for GERD. **C**, Diagnosis of the external test set based on probability scores. **D**, Probability score-based diagnosis of the equivocal test set.

Fig. 5: p(EoE) as a composite score to monitor therapy response. **A**, p(EoE) before and after steroid treatment. **B**, Examples of alterations in highly weighted EoE transcripts to steroid treatment. **C**, Heatmap of most significantly altered transcripts. ** p<0.01 *p<0.05 using ratio paired t-test.

Fig. 6: Serum IgE, esophageal IGHE transcript levels and local Th2-type inflammation. **A**, Patient Key. **B**, Correlation of serum IgE titers and esophageal IGHE transcript levels. Separation of EoE patients based on serum IgE and IGHE. **C**,

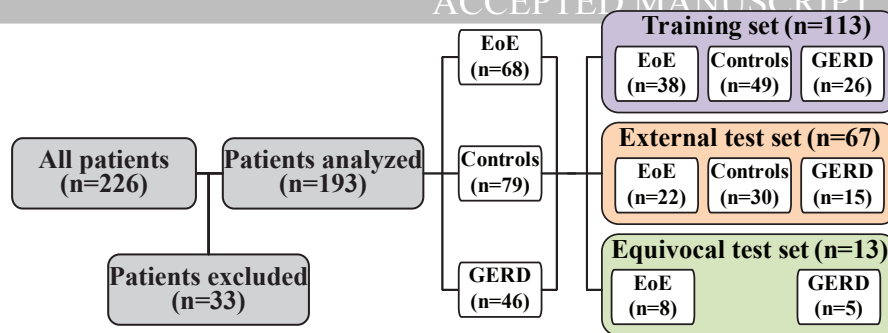
Eosinophil counts in IGHE-low (white circles normal serum IgE, black elevated IgE) and IGHE-high patients (orange circles normal IgE, red elevated IgE). **D**, Representative heat map of patients from the IGHE-high/serum-IgE-normal and IGHE-low/serum-IgE-elevated. Only most enriched genes are shown. **E**, Representative transcripts: FcεRIβ, CCL5, and IL13. **p<0.01 ***p<0.001; ****p<0.0001; as calculated by Dunn's multiple comparison test after Kruskal-Wallis test.

Fig. 7: IGHE score as a readout of esophageal allergic inflammation. **A**, Primary and secondary analysis loop. **B**, EoE patient groups stratified base on a composite score to distinguish IGHE-high and IGHE-low. **C**, ROC analysis. **D**) Correlation of IGHE-score with FcεRIβ mRNA. For patient key see Fig. 6A.

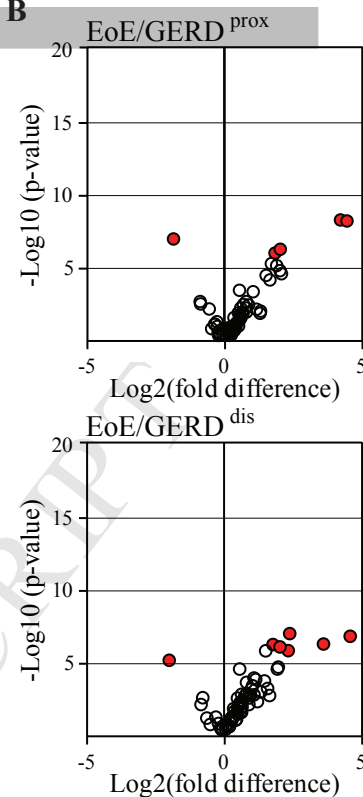
Table 1: Patient characteristics and cohort composition. p-values calculated by Chi-squared test.

Parameter	Learning Set				Test Set				Equivocal Set		
	EoE n	Control	GERD	P-value	EoE	Control	GERD	P-value	EoE	GERD	P-value
	38	49	26		22	30	15		8	5	
Age at diagnosis (in years; median, range)	10.31 (2.03-18.63)	10.08 (1.25-18.66)	10.79 (1.44-17.89)	0.948	10.78 (2.42-18.38)	10.02 (1.20-18.14)	13.63 (1.20-18.64)	0.207	7.32 (2.58-10.77)	12.10 (5.53-13.8)	0.052
Male gender	28/38 (74%)	18/49 (37%)	17/26 (65%)	<0.001	15/22 (68%)	14/30 (47%)	10/15 (67%)	0.225	5/8 (63%)	3/5 (60%)	0.929
Symptoms in the past year											
Dysphagia	21/38 (55%)	12/49 (24%)	5/26 (19%)	<0.001	11/22 (50%)	9/30 (30%)	4/15 (27%)	0.233	4/8 (50%)	1/5 (20%)	0.279
Food impaction	12/38 (32%)	2/49 (4%)	0/26 (0%)	<0.001	5/22 (23%)	0/30 (0%)	0/15 (0%)	0.003	2/8 (25%)	0/5 (0%)	0.224
Chest pain	2/38 (5%)	1/49 (2%)	2/26 (8%)	0.502	2/22 (9%)	2/30 (7%)	1/15 (7%)	0.939	1/8 (13%)	0/5 (0%)	0.411
Epigastric pain	2/38 (5%)	13/49 (27%)	5/26 (19%)	0.035	5/22 (23%)	9/30 (30%)	1/15 (7%)	0.208	0/8 (0%)	0/5 (0%)	NA
Reflux symptoms	10/38 (26%)	20/49 (41%)	18/26 (69%)	0.003	10/22 (45%)	17/30 (57%)	8/15 (53%)	0.723	2/8 (25%)	0/5 (0%)	0.224
Feeding difficulties	5/38 (13%)	7/49 (14%)	3/26 (12%)	0.946	4/22 (18%)	6/30 (20%)	1/15 (7%)	0.504	2/8 (25%)	2/5 (40%)	0.569
Vomiting	5/38 (13%)	14/49 (29%)	7/26 (26%)	0.206	6/22 (27%)	5/30 (17%)	3/15 (20%)	0.646	0/8 (0%)	0/5 (0%)	NA
Endoscopy											
Pallor	6/38 (16%)	1/49 (2%)	0/25 (0%)	0.010	1/22 (5%)	1/30 (3%)	2/15 (13%)	0.387	1/8 (13%)	0/5 (0%)	0.411
Edema	5/38 (13%)	1/49 (2%)	0/25 (0%)	0.028	3/22 (14%)	0/30 (0%)	0/15 (0%)	0.040	1/8 (13%)	1/5 (20%)	0.715
Loss of vascularity	16/38 (42%)	1/49 (2%)	5/25 (20%)	<0.001	6/22 (28%)	0/30 (0%)	2/15 (13%)	0.011	3/8 (38%)	0/5 (0%)	0.118
Furrowing	30/38 (79%)	3/49 (6%)	7/25 (28%)	<0.001	18/22 (82%)	2/30 (7%)	2/15 (13%)	<0.001	6/8 (75%)	3/5 (60%)	0.569
Exudate	15/38 (39%)	3/49 (6%)	1/25 (4%)	<0.001	7/22 (32%)	2/30 (7%)	0/15 (0%)	0.007	1/8 (13%)	1/5 (20%)	0.715
Allergic/atopic conditions											
Serum IgE levels (IU/mL median, range)	137 (4-1240)	56 (7-736)	25 (5-734)	0.300	107 (8-744)	37 (11-1524)	344 (11-970)	0.513	45 (7-2491)	744.5 (0-1489)	0.818
Eczema	10/37 (27%)	4/48 (8%)	5/26 (19%)	0.066	4/22 (18%)	4/29 (14%)	4/14 (29%)	0.504	3/6 (50%)	0/2 (0%)	0.206
Asthma	7/27 (19%)	3/48 (6%)	1/26 (4%)	0.013	5/22 (23%)	3/29 (10%)	4/14 (29%)	0.289	4/6 (67%)	1/2 (50%)	0.673
Allergic rhinoconjunctivitis	22/37 (59%)	3/48 (6%)	2/26 (8%)	<0.001	12/22 (55%)	3/29 (10%)	3/14 (21%)	0.002	3/6 (50%)	1/2 (50%)	1.000
Food allergy	11/37 (30%)	4/48 (8%)	1/26 (4%)	0.004	5/22 (23%)	5/30 (17%)	3/14 (21%)	0.879	4/6 (67%)	2/3 (67%)	1.000
RAST/skin prick test against food antigens	30/36 (83%)	7/16 (44%)	4/10 (40%)	0.003	14/18 (78%)	4/11 (36%)	5/8 (63%)	0.083	5/7 (71%)	1/2 (50%)	0.571
Tissue eosinophilia (peak value)											
Maximum (median, range)	47.5 (15-120)	0 (0-0)	2 (0-14)	<0.001	50 (15-150)	0 (0-0)	1 (0-8)	<0.001	40 (12-120)	20 (0-50)	0.134

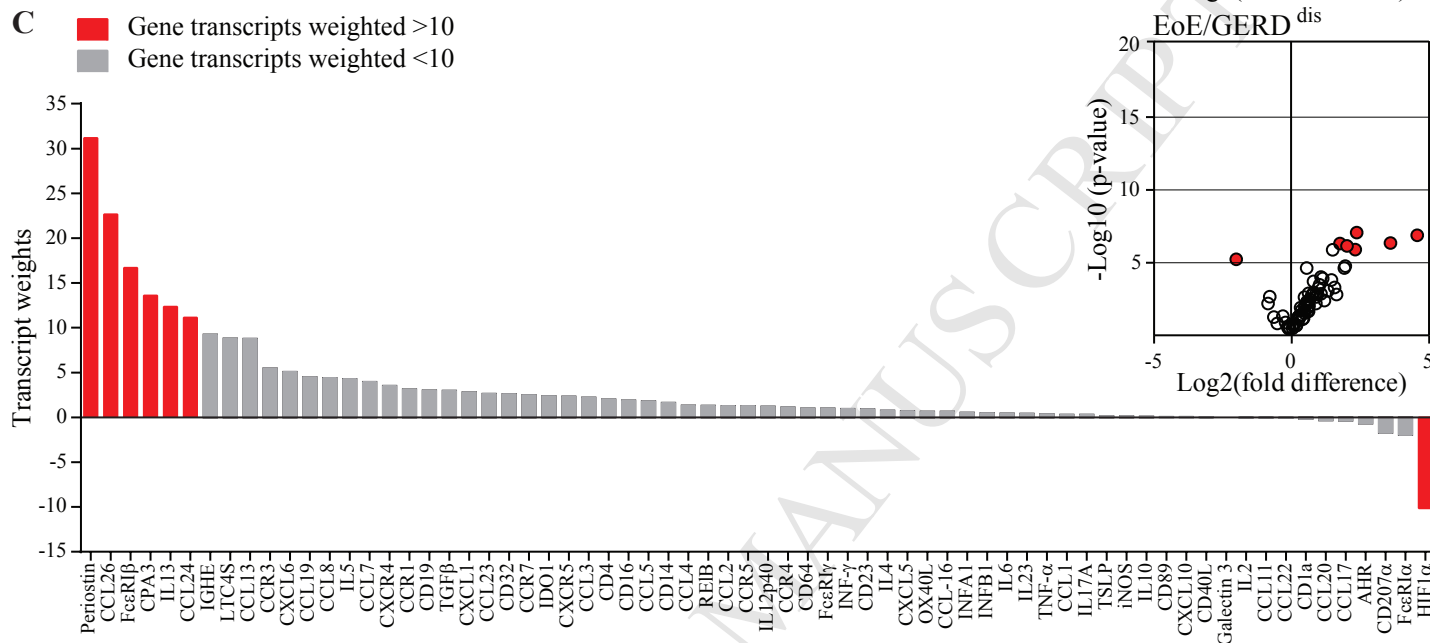
A



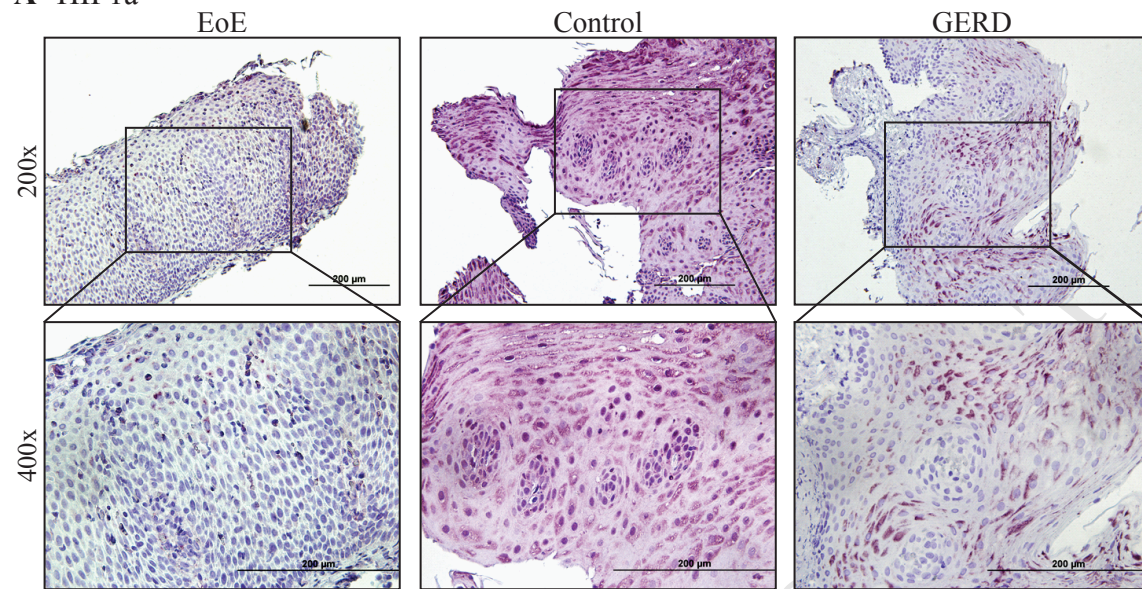
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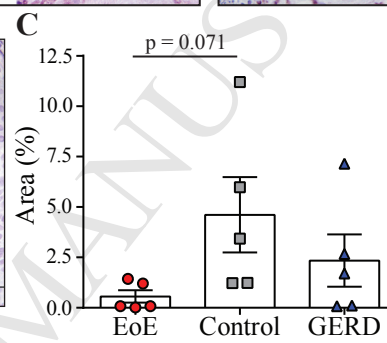
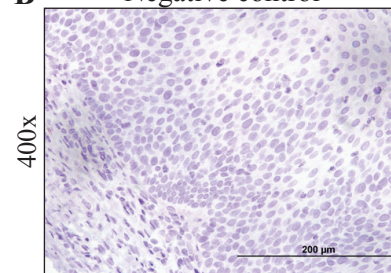
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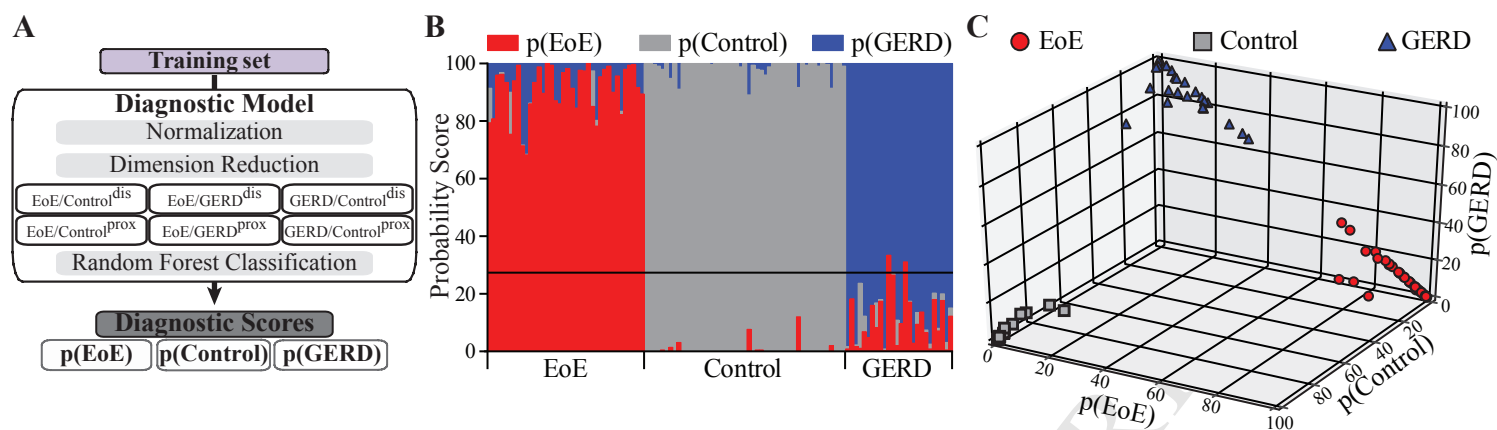


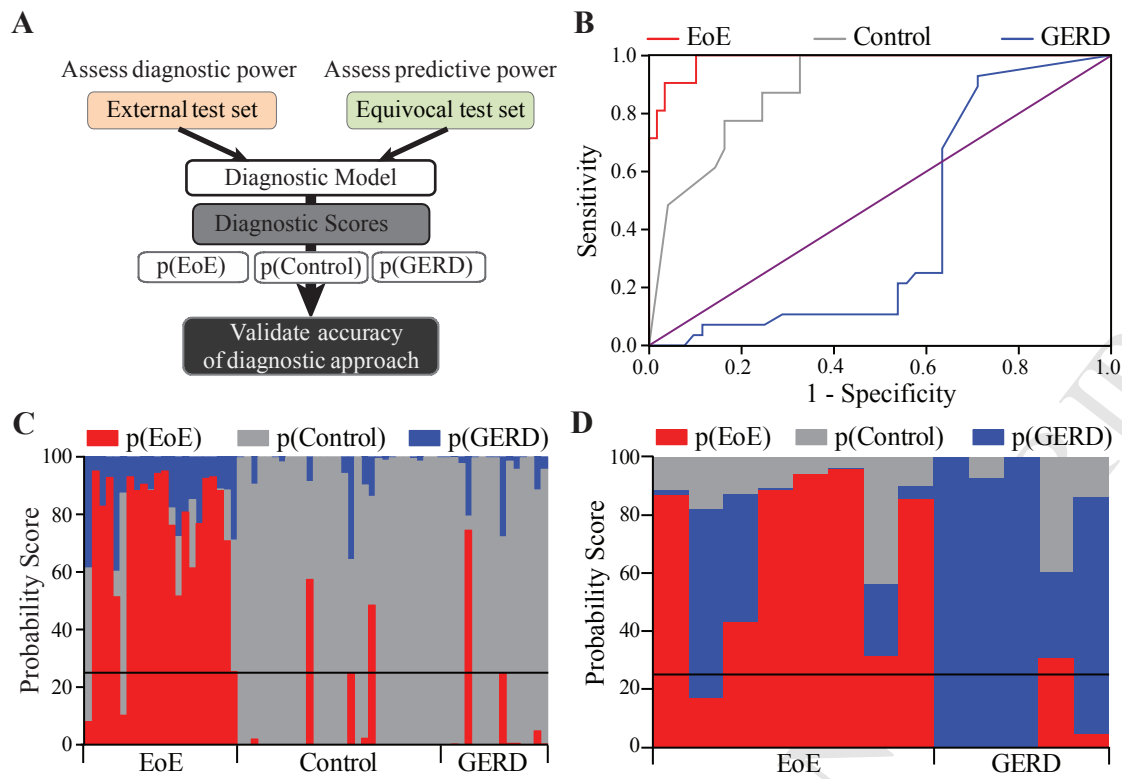
A HIF1a

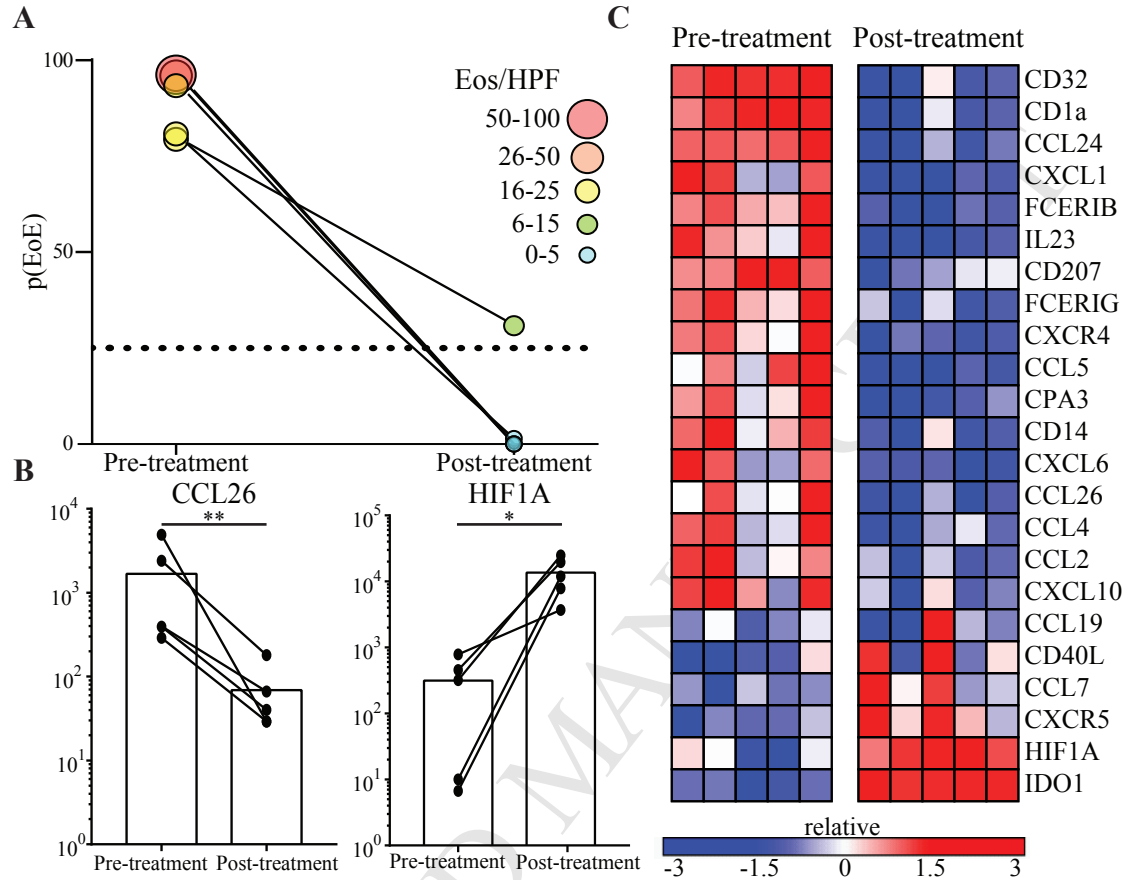


B Negative control





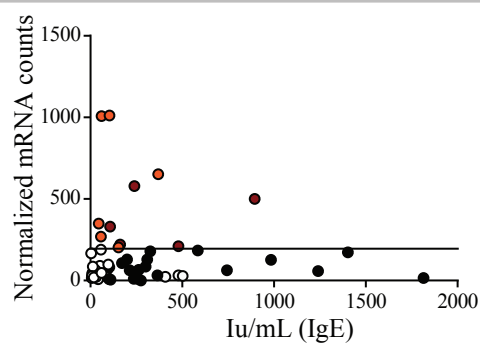




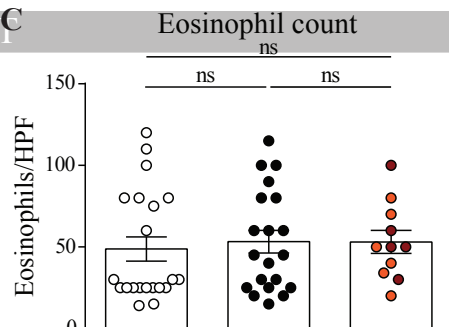
A

	Elevated Serum IgE	IGHE
●	+	High
○	-	High
●	+	Low
○	-	Low

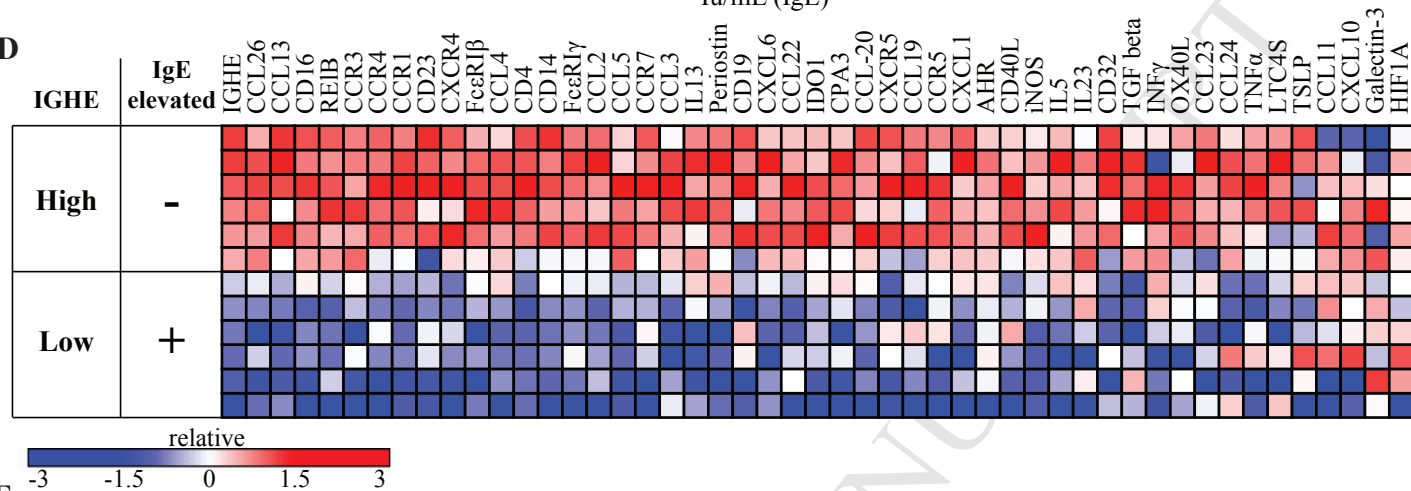
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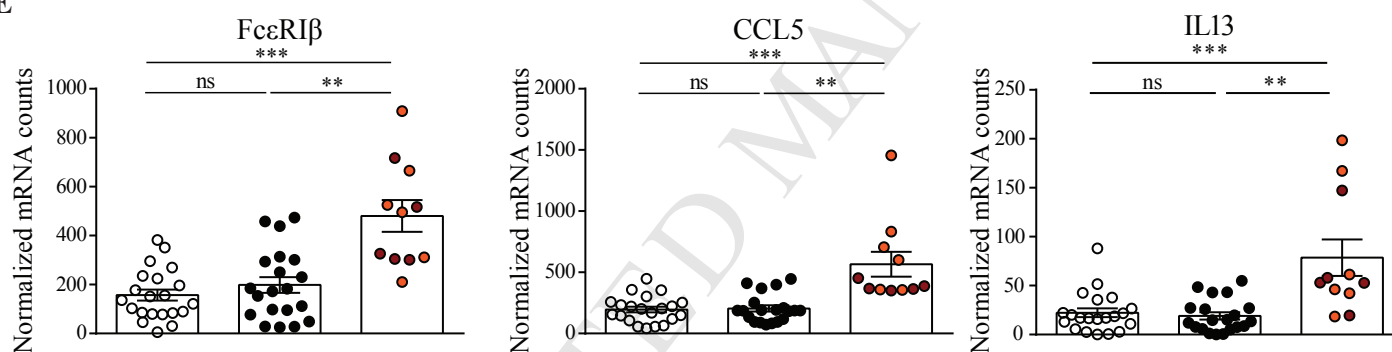
C



D



E



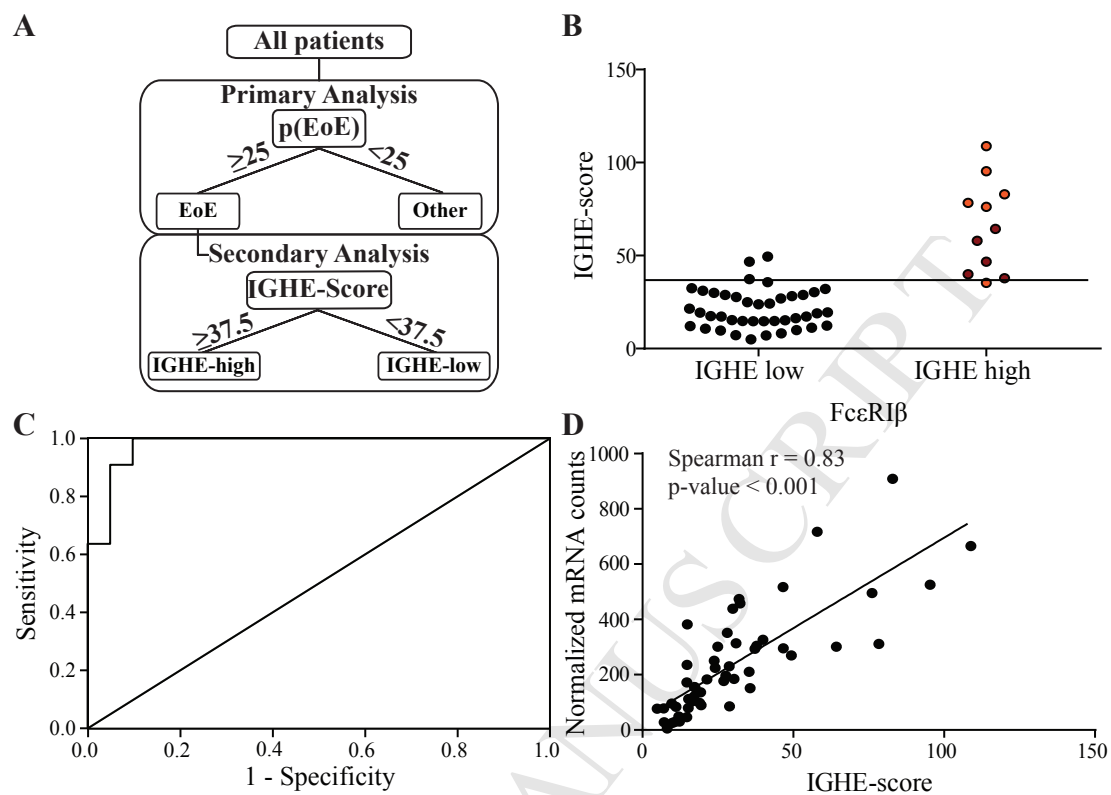


Fig. E1: Diagnostic classification by unsupervised machine learning. Principal component analysis plot of normalized transcript counts of the training set (n=113).

Fig. E2: Factors for the weighted component analysis. Volcano plots of normalized mRNA transcripts display fold difference (x-axis) and significance (y-axis) used for the calculation of the factors differentiating EoE and Controls as well as GERD and Controls in the proximal and distal esophagus in the training set (n=113). Red indicates transcripts with a weight >10.

Fig. E3: PCA of weighted factors. Plot of the training set after dimensionality reduction (n=113).

Fig. E4: Comparative ROC analysis of single transcripts for differentiating EoE from GERD and controls.

Fig. E5: Correlation of p(EoE) with disease severity markers. Correlation with eosinophil counts. Correlation with CCL26 mRNA levels in the training set (n=113).

Fig. E6: Eosinophil count and p(EoE) in atopic versus non-atopic patients. Comparison of A, Eosinophil counts/HPF and B, p(EoE) in patients with and without atopic comorbidities.

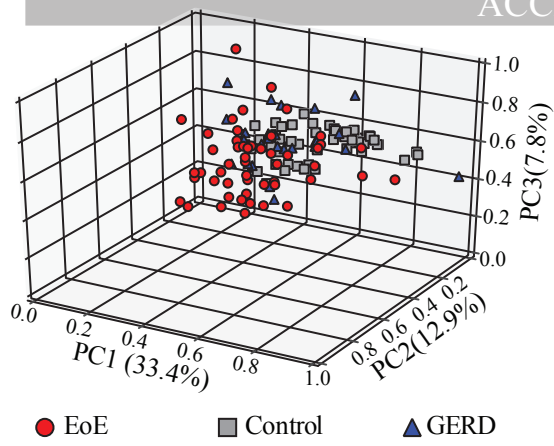
Fig. E7: p(EoE), Serum IgE, esophageal IGHE transcript levels and local Th2-type inflammation. A, Patient key. Quantification of B, p(EoE) C, IGHE transcript counts D, Serum IgE levels. E, Representative transcripts: IL5, CPA3, and CCL2. **p<0.01 ***p<0.001; ****p<0.0001; as calculated by Dunn's multiple comparison test after Kruskal-Wallis test.

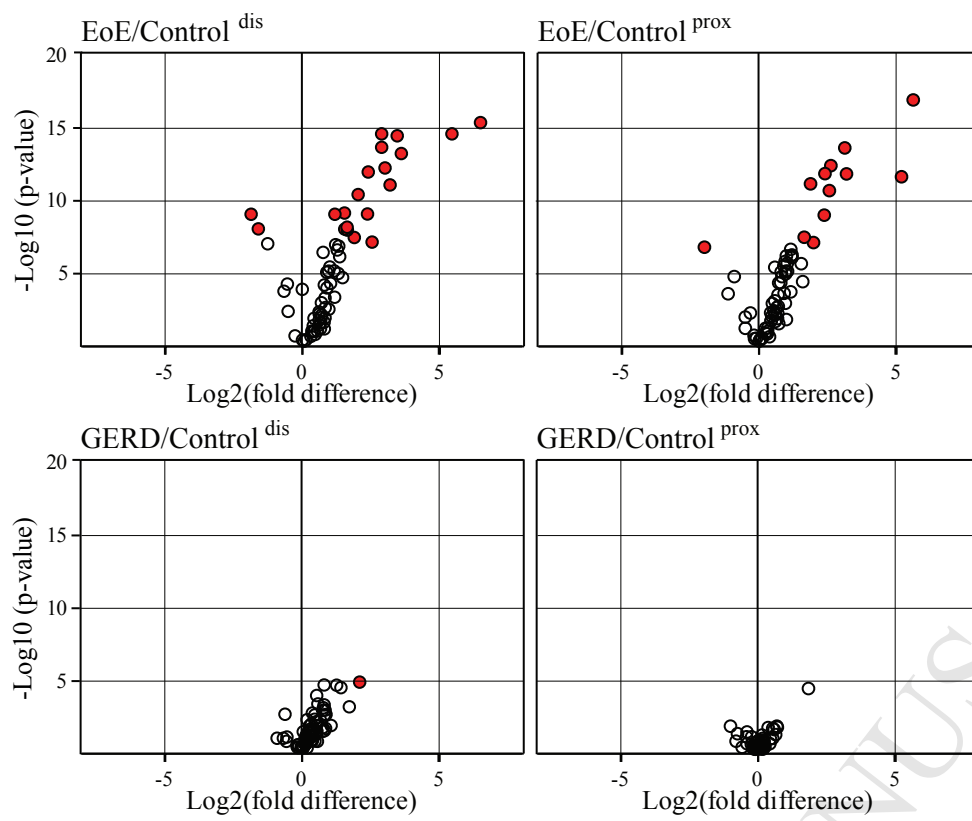
Table E1: Transcripts measured in probe set

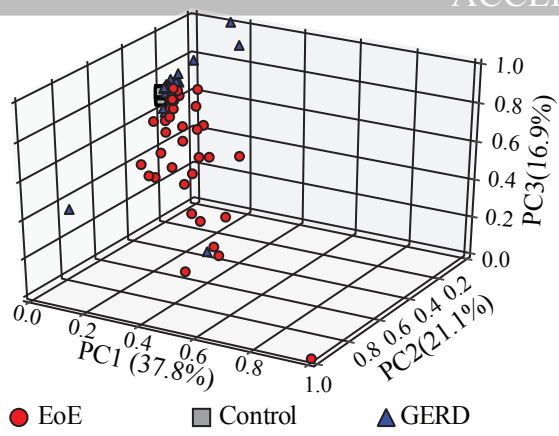
Table E2: Clinical characteristics of patients used for tissue allergy analysis

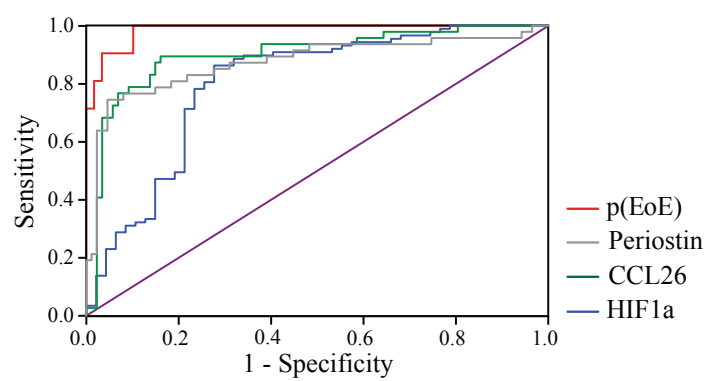
ACTB	CCL3	CD32	GAPDH	IL6
AHR	CCL4	CD4	HIF1A	INFA1
CPA3	CCL5	CD40L	HPRT1	INFB1
CCL1	CCL7	CD64	Hsp90ab1	IFNg
CCL11	CCL8	CD89	IDO1	iNOS
CCL13	CCR1	CXCL1	IGHE	CD207
CCL16	CCR3	CXCL10	IL10	LTC4S
CCL17	CCR4	CXCL5	IL12 p40	OX40L
CCL19	CCR5	CXCL6	IL13	POSTN
CCL2	CCR7	CXCR4	IL17	REIB
CCL20	CD14	CXCR5	IL17A	RPL13A
CCL22	CD16	FcER1a	IL2	TGFb
CCL23	CD19	FcER1b	IL23	TNFa
CCL24	CD1a	FcER1g	IL4	TSLP
CCL26	CD23	Gal3		

Parameter	Tissue Allergy Set			P-value
	IGHE high	IgE high	IgE low	
n	11	20	21	
Age at diagnosis (in years; median, range)	10.07 (2.03-15.36)	8.38 (2.91-17.67)	10.26 (2.88-18.63)	0.454
Male gender	4/11 (36%)	17/20 (85%)	12/21 (57%)	0.020
Symptoms in the past year				
Dysphagia	6/11 (55%)	11/20 (55%)	10/21 (48%)	0.877
Food impaction	2/11 (19%)	3/20 (15%)	5/21 (24%)	0.770
Chest pain	1/11 (9%)	0/20 (0%)	0/21 (0%)	0.150
Epigastric pain	3/11 (27%)	2/20 (10%)	2/21 (10%)	0.319
Reflux symptoms	4/11 (36%)	4/20 (20%)	10/21 (48%)	0.176
Feeding difficulties	1/11 (9%)	3/20 (15%)	2/21 (10%)	0.826
Vomiting	2/11 (19%)	6/20 (30%)	1/21 (5%)	0.102
Endoscopy				
Pallor	3/11 (27%)	4/20 (20%)	1/21 (5%)	0.188
Edema	2/11 (19%)	3/20 (15%)	2/21 (10%)	0.767
Loss of vascularity	3/11 (27%)	8/20 (35%)	2/21 (10%)	0.078
Furrowing	9/11 (82%)	16/20 (80%)	14/21 (67%)	0.518
Exudate	3/11 (27%)	5/20 (25%)	8/21 (38%)	0.636
Allergic/Atopic conditions				
Serum IgE levels (IU/mL median, range)	153 (45-895)	287 (97-1815)	42 (4-503)	0.002
Eczema	4/11 (26%)	2/19 (11%)	7/21 (33%)	0.164
Asthma	1/11 (9%)	4/19 (21%)	6/21 (29%)	0.444
Allergic rhinoconjunctivitis	8/11 (73%)	15/19 (79%)	10/21 (48%)	0.096
Food allergy	6/11 (55%)	8/19 (42%)	1/21 (5%)	0.004
RAST/skin prick test against food antigens	10/11 (91%)	19/19 (100%)	14/21 (67%)	0.012
Tissue eosinophilia (peak value)				
Proximal (median, range)	40 (20-70)	20 (0-100)	9.5 (0-110)	0.273
Distal (median, range)	50 (20-100)	25 (2-90)	30 (14-120)	0.326
Maximum (median, range)	50 (20-100)	45 (15-115)	30 (14-120)	0.326









	AUC	Sensitivity	Specificity
p(EoE)	0.985 ± 0.013	0.91	0.93
Periostin	0.875 ± 0.037	0.75	0.95
CCL26	0.899 ± 0.031	0.89	0.84
HIF1a	0.799 ± 0.044	0.86	0.72

